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ABNORMAL SULFATE METABOLISM IN A HEREDITARY DEMYELINATING NEUROPATHY

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SUMMARY

Trembler mice are affected by dominantly inherited neuropathy. Total lipid content and sulfatides were decreased in peripheral nerves from 15-day-old mutants. The proportion of sulfatides in per cent of total lipids was similar in control and Trembler nerves. The specific activity of ceramide galactosyltransferase, the enzyme responsible for the synthesis of cerebrosides, was 36 and 13% of controls, in young and adult Trembler nerves, respectively. In contrast, cerebroside sulfotransferase activities were increased by 257 and 172%, in young and adult Trembler sciatic nerves, respectively. No activator or inhibitor effect could be demonstrated. In Trembler PNS, K_m, V_{max} and heat sensitivity of CST differed from controls. Low levels of substrate and high arylsulfatase A activity (218% of controls) could explain the lack of sulfatide accumulation. The increased in vivo sulfate and galactose incorporation into non-lipidic material could reflect the overproduction of endoneurial and perineurial connective tissue, whereas the high turnover rate of sulfatides could be correlated with intense demyelination and remyelination observed in Trembler PNS.

INTRODUCTION

Trembler mice are affected by a dominantly inherited neuropathy[9]. Peripheral nerves of Trembler mutants show morphological evidence of recurrent demyelination and remyelination superimposed upon hypomyelination[2,14–16]. The protein composi-

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tion of Trembler sciatic nerves and of purified myelin is consistent with marked demyelination[19]. The lipid composition of sciatic nerves from Trembler mice showed a 66% decrease in total lipids affecting in a similar way each individual lipid and a 5-fold increase of cholesterol esters suggesting active demyelinating processes[13]. Using an elegant grafting technique, Aguayo et al.[1] proved unequivocally that the Trembler neuropathy is due to a primary disorder of Schwann cells. The two biochemical studies mentioned above did not reveal any specific anomaly in Trembler nerves but confirmed hypomyelination and demyelination processes. In the present investigation, we studied the incorporation of radioactive sulfate into sulfatides and non-lipidic material. In addition enzymes involved in the metabolism of sulfatides were measured and some of the characteristics of galactocerebroside sulfotransferase (phosphoadenosine phosphosulfate: galactosylceramide sulfotransferase, cerebroside sulfotransferase or CST, EC 2.8.2.11) were analyzed in Trembler and control peripheral nervous tissue. A preliminary report of this work was presented in abstract form[20].

MATERIAL AND METHODS

Animals and tissue preparation

Trembler and control mice were bred in these laboratories from stock originally obtained from the Institute of Animal Genetics, Edinburgh, U.K. For some experiments, animals were anesthetized with ether and bilateral injections of radioactive sulfate (10 μCi of $Na_2{}^{35}SO_4$, 10–1000 mCi/mmol sulfur) or galactose (10 μCi of D-[^{3}H(G)]galactose, 1–5 Ci/mmol) dissolved in 5 μl physiological saline were made at several sites into the exposed sciatic nerves. Animals were killed 16 h later. The whole brain above the foramen magnum was quickly removed and chilled in ice-cold saline and stored at —70 °C until analysis. The sciatic nerves between the spinal origin and the knee were removed and carefully freed of surrounding connective tissues. Although the mutant animals have a smaller body weight ($P < 0.001$) than their control littermates, no significant weight difference was found for sciatic nerves between the two groups. The sciatic nerves from several mice were rinsed in ice-cold saline, pooled and stored frozen (—70 °C) until used for lipid analysis or enzymatic determination. The tissue was homogenized at 0–4 °C in 0.32 M sucrose using an all-glass Ten Broeck homogenizer.

Lipid analysis

The sciatic nerves were homogenized in 0.32 M sucrose and 10 μl were used to assay for protein[17], the rest was lyophilized after determination of its volume. Lipids were extracted with chloroform–methanol (2:1, v/v), partitioned[10] and the lipid extract was concentrated under a stream of nitrogen. Lipid aliquots were used for total lipid determination by gravimetry, sulfatide colorimetric determination[11]. [^{3}H]total lipids and [^{35}S]sulfatides were counted for radioactivity by liquid scintillation spectrometry. Controls showed that after separation on one-dimensional thin-layer chromatography, 90% of the total radioactive sulfate was recovered in the sulfatide spot.

Enzyme assays

Cerebroside sulfotransferase (EC 2.8.2.11, CST) was determined using two different assay techniques[4,24]. UDP-galactose:ceramide galactosyltransferase (EC 2.4.1.45, CGalT) was measured using an improved procedure that utilizes appropriate acceptor-incorporated liposomes[5]. 2',3'-Cyclic nucleotide 3'-phosphodiesterase (EC 3.1.4.37, CNP) and arylsulfatase A (EC 3.1.6.1, ASA) were assayed using the methods of Kurihara and Tsukada[12] and Baum et al.[3], respectively.

RESULTS

Total lipids, sulfatides and incorporation of [^{35}S]sulfate

These experiments were performed in 15-day-old mice when demyelination is not too prevailing in Trembler mutants[19]. Total lipids in Trembler sciatic nerves were 24% of controls. The amount of sulfatides showed a similar reduction (Table I) and expressed in per cent of total lipids there was no difference between mutants and controls. The values measured were similar to those reported by Larrouquère-Régnier et al.[13] using a different technique.

The amounts of total protein and residual proteins (proteins measured after delipidation of the tissue) in Trembler sciatic nerves were slightly increased but the difference was not statistically significant (Table I). Although the amount of ^{35}S incorporated into sulfatides per nerve or per mg of protein was similar in mutants and controls, the sulfatide specific radioactivity was 4 times higher in the mutants (Table I). The rate of incorporation of ^{35}S into non-lipidic material (chloroform–methanol insoluble material: proteins, glycoproteins and mucopolysaccharides) was increased in Trembler nerves by a factor of 2.4 (Table I). The incorporation of radioactive sulfate into the major PNS myelin protein PO was not increased (Matthieu

TABLE I

In vivo incorporation of [^{35}S]sulfate into PNS lipids and non-lipidic material

Time of incorporation: 16 h. Age of animals: 15 days. Mean ± S.E.M., Student's *t*-test.

	Control	*Trembler*	*P*
Total proteins (μg/nerve)	213 ± 13.0 (14)	256 ± 18.2 (14)	n.s.
Residual proteins* (μg/nerve)	154 (2)	187 (2)	
Total lipids (mg/100 mg fresh weight	8.5 ± 1.2 (4)	2.0 ± 0.3 (4)	<0.01
Sulfatides (nmol/100 mg fresh weight	4.3 ± 0.6 (4)	1.3 ± 0.1 (3)	<0.01
(nmol/100 mg total lipids)	5.1 ± 0.4 (4)	6.4 ± 0.7 (3)	n.s.
[^{35}S]Sulfatides (dpm/mg prot. × 10^{-3})	34.3 ± 3.0 (6)	41.4 ± 4.9 (6)	n.s.
(dpm/nmol sulfatide × 10^{-6})	0.5 (6)	2.1 (6)	
[^{35}S]Non-lipidic material (dpm/mg total prot. × 10^{-3})	15.8 ± 0.8 (6)	38.6 ± 4.4 (6)	<0.001

* After chloroform–methanol extraction.

TABLE II

In vivo incorporation of [^{3}H]galactose into PNS lipids and non lipidic material

Time of incorporation: 16 h. Age of animals: 15 days. Mean ± S.E.M., Student's *t*-test.

	Control	*Trembler*	*P*
[^{3}H]Galactolipids (dpm/mg prot. 10^{-3})	39.08 ± 2.75 (4)	17.90 ± 1.92. (4)	<0.001
[^{3}H]non-lipidic material (dpm/mg prot. 10^{-3})	11.16 ± 1.27 (4)	24.42 ± 2.09 (6)	<0.01

et al., unpublished results). In pulse-chase experiments, performed in vitro, a higher incorporation peak of radioactive sulfate was followed by a rapid decrease of radioactivity in the sulfatides of Trembler sciatic nerves when compared to control nerves (results not shown). The incorporation of [^{3}H]galactose into total lipids was significantly decreased in Trembler sciatic nerves (Table II) in accordance with the low lipid content of the tissue. In contrast, the incorporation of [^{3}H]galactose into non-lipidic material was increased (Table II).

Specific activities of CST, CGalT, ASA and CNP

Using two different techniques, the specific activity of CST, the enzyme responsible for the synthesis of sulfatides was increased in Trembler sciatic nerves at two different ages in the young animals (Table III). In contrast, the specific activity of CGalT in Trembler mice was low at 15 days and the difference with controls increased in young adults (Table III). The activity of ASA, the enzyme involved in the first hydrolytic step of sulfatides, was increased (Table III) to levels observed during Wallerian degeneration (Matthieu et al., in preparation). The specific activity of CNP, a myelin marker enzyme in CNS, probably associated with Schwann cells in PNS[21], was significantly decreased in Trembler sciatic nerves. The specific activity of these

TABLE III

Enzyme specific activities in Trembler sciatic nerves

Mean values of 3 to 7 experiments expressed in per cent of controls.

CGluT, UDP-glucose: ceramide glucosyltransferase; CGalT, UDP-galactose: ceramide galactosyltransferase; CST, phosphoadenosine phosphosulfate:galactosylceramide sulfotransferase; ASA, arylsulfatase A; CNP, 2',3'-cyclic-nucleotide 3'phosphodiesterase. n.d., not determined.

Enzyme	*15 days*	*4–6 weeks*
CGlut	94*	84*
CGalt	36***	13***
CST	257**	172**
ASA	218**	n.d.
CNP	66**	43**

*, Not significant; **, $P < 0.01$; ***, $P < 0.001$ (Student's *t*-test).

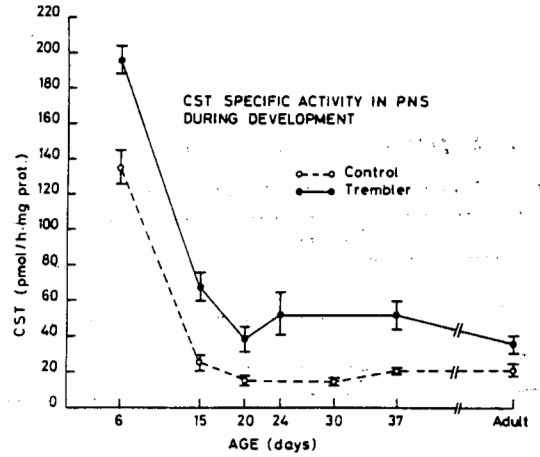

Fig. 1. Activity of galactocerebroside sulfotransferase (CST)[4] in sciatic nerves during development. Mean values ± S.E.M. from 4–9 separate experiments. Open circles, controls; closed circles, Trembler mice.

enzymes did not show any significant difference when determined in CNS homogenates between Trembler and control mice at two different ages.

Developmental changes of CST in sciatic nerves

The specific activity of CST was measured at different ages during development in both Trembler and control mice (Fig. 1). The developmental changes showed the maximum specific activity of CST at the earliest age with a sharp drop between 6 and 15 days. After 15 days of age, the specific activity of CST did not show any significant changes both in Trembler or in control mice. However, the specific activity of the enzyme in the Trembler nerves remained higher than in control nerves throughout development.

Some characteristics of CST in Trembler and control mice

In order to find an explanation for the increased activities of CST measured in PNS, experiments were performed to demonstrate the presence of natural activators (Trembler) or inhibitors (control). Sciatic nerve homogenates were used as a source of enzyme and by mixing aliquots of Trembler or control homogenates, no activation or inhibition could be demonstrated (Table IV).

TABLE IV

Cerebroside sulfotransferase activity in sciatic nerves from Trembler mice—mixing experiments

Activity expressed in dpm. This representative experiment was chosen from among three.

Homogenate	*Observed*	*Calculated*
Trembler	441	—
Control	282	—
Trembler + control	743	723
Boiled Trembler	20	—
Boiled control	41	—
Boiled Trembler + control	258	302
Boiled control + Trembler	481	482

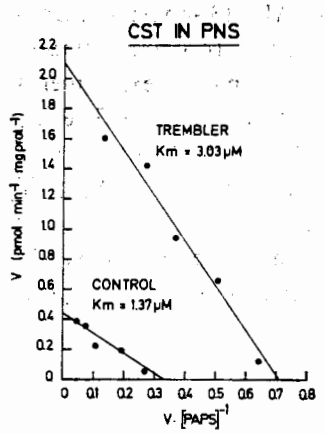

Fig. 2. Eadie–Hofstee plot of a representative experiment obtained from varying concentrations of [^{35}S]PAPS. The mean K_m values obtained from 4–6 separate experiments are indicated for control and Trembler PNS tissue. CST activities were measured according to Tennekoon and McKhann[24].

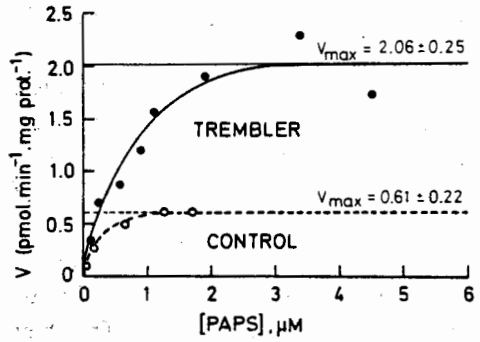

Fig. 3. Determination of V_{max} with varying concentrations of [^{35}S]PAPS. Mean values ± S.E.M. obtained from 4–6 separate experiments are indicated above the curve of a representative experiment.

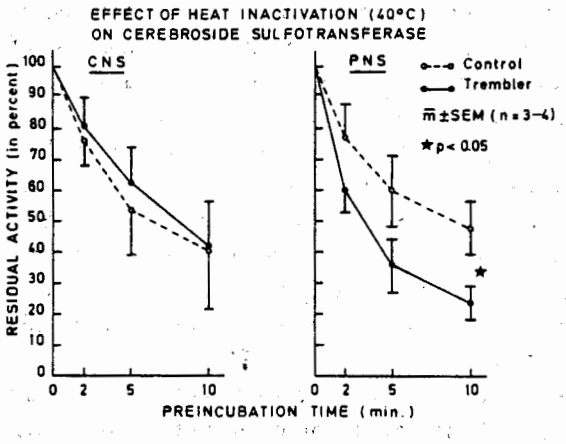

Fig. 4. Heat sensitivity of galactocerebroside sulfotransferase from CNS (left panel) and PNS (right panel). Mean values ± S.E.M. from 3–4 separate experiments. Open circles, control; closed circles, Trembler mice.

Eadie–Hofstee plots of data (Fig. 2) using the Tennekoon and McKhann technique[24] obtained from varying concentrations of [^{35}S]PAPS (3′-phosphoadenosine 5′-phosphosulfate) gave a K_m of 1.37 μM and 3.03 μM for control and Trembler sciatic nerves, respectively. These values obtained from 4–6 separate experiments showed less than 10% variation and are significantly different ($P < 0.001$). The K_m value measured in control sciatic nerves was lower than in CNS (2.45 μM $\pm$ 0.4, n = 4) where no difference was found between control and Trembler mice. The V_{max} in Trembler PNS was 3.4 times higher than in controls (Fig. 3).

The effect of heat inactivation was similar in control and Trembler CNS. In contrast, CST in Trembler PNS was more heat sensitive than that from controls (Fig. 4).

DISCUSSION

Trembler mice are affected by recurrent demyelination and remyelination superimposed upon hypomyelination[2,14–16]. The protein[19] and lipid analysis[13] is consistent with hypo- and demyelinating processes. The present study gives evidence of an abnormal sulfate metabolism in Trembler sciatic nerves, whereas no anomaly could be demonstrated in the CNS.

The increased specific activity of CST, revealed with two different techniques, confirmed the increased rate of radioactive sulfate incorporation into cerebrosides. This and the in vitro study makes unlikely the possibility that a small sulfate pool could be responsible for the high ^{35}S incorporation rate into sulfatides. The sulfation of PO (Matthieu et al., in preparation) was not increased.

Low concentrations of cerebrosides[13], supported by low galactosyltransferase activities and high arylsulfatase A activity, could further explain the lack of sulfatide accumulation. These findings suggest an increased sulfatide turnover in Trembler PNS. Non-lipidic substrates showed increased incorporations of radioactive sulfate and galactose. This could be the biochemical expression of the overproduction of endoneurial and perineurial connective tissue, particularly basement membranes observed in Trembler nerves[2], in which the number of Schwann cells and fibroblasts is increased[16]. This increased number of Schwann cells did not involve an increase of galactosyltransferase or CNP activities, another indication of an abnormal function reported previously[1]. Since axons contain considerable amounts of sulfatides[7], an accelerated sulfate turnover in the axonal compartment is another possibility. Nevertheless, transplantation experiments[1] have shown that the axons from Trembler mice are normal and we found normal CST and ASA specific activities in the CNS of these mutants. Therefore, we consider this possibility as unlikely. Increased CST specific activities cannot be explained by a lack of product inhibition since the mixing experiment was negative. Furthermore, with similar low cerebroside levels the specific activity of CGalT was not increased, but significantly reduced.

The developmental curve of CST in control PNS differs significantly from that reported for the CNS[23]. This could be explained by the onset of myelination which occurs at an earlier age in peripheral nerves than in the CNS and agrees with the

maximum in vivo sulfatide synthesis which was reported to occur at 10 days of age in rats[25].

Important kinetic differences for cerebroside sulfotransferase were observed between Trembler and control peripheral nervous tissue suggesting that the mutation affects the enzyme protein structure. CST in CNS, which seems to be under a different genetic control than that in the PNS[18], is unaffected in Trembler mice. Trembler CST seems to be present in high concentration in PNS since, in spite of a higher K_m it exhibits high specific activity. In experimental demyelination and remyelination (Matthieu et al., in preparation) no increase in CST activity was observed and the data presented suggest that the abnormal kinetic characteristics of CST and abnormal sulfate metabolism could be specific for this murine demyelinating neuropathy. Nevertheless, we cannot exclude the possibility that the high activity of CST in Trembler PNS is non-specific since higher incorporation of ^{35}S in non-lipidic material was observed. It would be of interest to measure the CST activity in human neuropathies to see whether this test could be of help to sort out the different clinical forms, since previous reports[8,22] suggested an abnormal sulfatide metabolism in sural nerves of patients with chronic onion bulb neuropathies.

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